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(54) Title: METHOD FOR SEQUENCING NUCLEIC ACIDS USING MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

(57) Abstract

The invention provides an analytical method for determining the nucleotide sequence of nucleic acid analytes, including chemically modified oligonucleotides. This new method utilizes matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) with delayed pulsed ion extraction.

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METHOD FOR SEQUENCING NUCLEIC ACIDS USING MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to the determination of nucleotide sequences for nucleic acids and their analogs.

Brief Summary of the Related Art

Determination of nucleotide sequences for various nucleic acids has become a fundamentally important analytical step for numerous molecular biology and biomedical applications. Consequently, a variety of methods have been developed to facilitate such nucleotide sequence determinations. Maxam and Gilbert, Proc. Natl. Acad. Sci. USA 74: 5460 (1977) discloses a chemical degradation approach for DNA sequence determination. Sanger *et al.*, Proc. Natl. Acad. Sci. USA 74: 5463 (1977), discloses a chain termination method using complementary strand primer extension to determine DNA sequences. Each of these methods utilizes four separate reaction mixtures to create a nested set of fragments differing by a single nucleotide in length and representing a complete nucleotide sequence, followed by resolution of the fragments based on their size to determine the order of the fragments and hence the nucleotide sequence. Both of these procedures take from numerous hours to days to perform, and neither is effective for determining the nucleotide sequence for certain analogs of DNA.

These popular approaches to nucleotide sequence determination are limited by their time consuming methodologies and by their inapplicability to certain types of nucleic acid analytes. For example, small synthetic oligonucleotides have recently become of interest as tools in molecular biology experiments, as well as for use in the antisense therapeutic approach to disease treatment. Correct sequences are necessary to the efficacy and safety of such oligonucleotides, and effective and rapid

analytical approaches are needed for quality control. This class of compounds presents three special problems for traditional sequence determination approaches. First, quality control procedures are needed which are more rapid than the traditional approaches. Second, the oligonucleotides are generally short, often in the range of from about 15 to about 35 nucleotides in length. As a consequence, traditional sequencing approaches result in the loss of much sequence information and thus provide only incomplete information about the nucleotide sequence of the oligonucleotides. Finally, many of these oligonucleotides have either modified internucleoside linkages or substitution at the 2' position of the ribose to improve their properties as potential therapeutic agents. U.S. Patent No. 5,220,007 discloses chimeric oligonucleotides having regions of oligonucleoside phosphodiester or phosphorothioate alongside regions of oligonucleoside alkylphosphonate or phosphoramidate. PCT publication WO94/02498 discloses hybrid oligonucleotides having DNA regions alongside 2'-substituted RNA regions. Uhlman and Peyman, Chemical Reviews 90: 544 (1990), discloses oligonucleotides having a variety of modifications along the internucleoside linkages, sugar residues or nucleoside bases.

Recently, some approaches have been developed to address these special problems. U.S. Patent No. 5,403,709 discloses a method for sequencing oligonucleotides using another oligonucleotide as an extension and a third, bridging oligonucleotide to hold the first two together for ligation. Conventional primer extension is then used to create a complement for sequencing. This approach requires some advance knowledge of a portion of the sequence of the analyte oligonucleotide. U.S. Patent No. 5,525,470 discloses a similar approach which avoids the need for such advance knowledge by utilizing RNA ligase to couple the analyte and extension oligonucleotides. Both of these approaches can be used with some modified oligonucleotides, especially with those having phosphodiester or phosphorothioate internucleotide linkages. Both, however, are time-consuming and limited to analyte oligonucleotides which can act as templates for the polymerase enzyme used to synthesize their complement.

Recently, Nordhoff *et al.*, J. Mass Spec. 30: 99 (1995) disclosed a direct sequencing method for oligonucleotides using infrared matrix-assisted laser desorption/ionization mass spectrometry. This method utilized time-of-flight analysis of prompt fragment ions generated by infrared laser desorption/ionization. Fragments were reported to be generated by consecutive cleavage of the deoxyribose phosphate backbone at apurinic sites. Unfortunately, such cleavage does not occur at T residues, due to failure to remove the thymidine base, and may not occur in RNA due to stabilization of the glycosidic bond. In addition, there is a problem with the prompt fragment ions overlapping with "post-source decay" fragment ions (PSD ions) from metastable decays occurring in the field-free region of the time-of-flight analyzer.

Brown and Lennon, Anal. Chem. 67: 3990 (1995) discloses sequence-specific fragmentation of matrix-assisted laser-desorbed protein/peptide ions and detection of the fragments using time-of-flight mass spectrometry with delayed pulsed ion extraction. The delayed pulsed ion extraction is used to reduce the generation of PSD ions by expanding the desorbed neutral plume during the extraction delay period, thereby avoiding energetic collisions believed to play a role in the generation of PSD. The technique was found to be applicable to small peptides and one special case larger protein.

There remains a need for more rapid approaches for determining the nucleotide sequence of nucleic acid analytes. In addition, there is a need for new sequencing approaches that address the special sequence determination problems presented by synthetic oligonucleotides. Ideally, such a new approach should be rapid and universal, should act directly on the nucleic acid analyte, and should be applicable to any chemically modified oligonucleotides.

BRIEF SUMMARY OF THE INVENTION

The invention provides a universal analytical method for determining the nucleotide sequence of nucleic acid analytes, including any chemically modified oligonucleotides. This new method utilizes matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) with delayed pulsed ion extraction. This method is extremely rapid and acts directly on the nucleic acid analyte, creating in-source fragmentation (ISF) products which are used to determine the sequence of the nucleic acid analyte. Such ISF products have earlier also been known as prompt fragmentation ions, and such terms can be used interchangeably although in-source fragmentation product is the currently preferred term and will be used throughout herein. It is effective for a variety of nucleic acid analytes, including any chemically modified oligonucleotides which have not previously been successfully sequenced.

The invention provides a method for determining the nucleotide sequence of nucleic acid analytes comprising providing a suitable target for matrix-assisted laser desorption/ionization, such target comprising a nucleic acid analyte in a matrix suitable for such matrix-assisted laser desorption/ionization; irradiating the target with laser irradiation suitable for creating ISF products from the nucleic acid analyte; delaying pulsed ion extraction for a time sufficient to reduce formation of PSD ion fragments; ordering the ISF products according to molecular weight by time-of-flight mass spectrometry; and determining the nucleotide sequence of the nucleic acid analyte by comparing the molecular weights of the ordered ISF products.

Preferably, the suitable target will include a solid support, such as the commercially available solid supports provided by Bruker Daltonics, formerly Bruker Analytical Systems (Billerica, MA) or PerSeptive Biosystems (Cambridge, MA). Generally, the target is prepared by applying to the solid support a solution comprising the nucleic acid analyte and a matrix suitable for matrix-assisted laser desorption/ionization (MALDI), then allowing the solution on the solid support to evaporate to dryness. The nucleic acid analyte can be a naturally occurring or

synthetic polynucleotide or oligonucleotide, including oligonucleotides having chemically modified internucleoside linkages, sugar backbones or nucleoside bases. The suitable matrix can be selected from the matrices known in the art to be suitable for MALDI, and includes a cation exchange resin, preferably in ammonium form. Preferred matrices include, without limitation, 3-hydroxypicolinic acid, 3-hydroxypicolinic acid/picolinic acid, 3-hydroxypicolinic acid/sucrose, 2,5-dihydroxybenzoic acid, sinapinic acid, alpha-cyano-4-hydroxy-cinnamic acid, anthranilic acid, 6-aza-2-thiothymine, nicotinic acid, 2,4,6-trihydroxybenzoic acids, 2,4,6-trihydroxyacetophenone and combinations of the same. Other suitable matrices are identified in Fitzgerald *et al.*, Anal. Chem. 65: 3204 (1993). In one particularly preferred embodiment, the matrix is 3-hydroxypicolinic acid/N-(3-indolylacetyl)-1-leucine (3-HPA/IAL).

The target can be irradiated with any of a variety of laser irradiation sources. Preferred laser irradiation sources include, without limitation, UV lasers, visible lasers and IR lasers. Particularly preferred UV lasers include a 337 nm N_2 laser, a 266 nm laser, a 193 nm laser and a 355 nm laser.

The pulsed ion extraction is delayed for a time sufficient to increase mass resolution of the ISF products and to reduce PSD ion formation sufficiently to improve the profile of the time-of-flight mass spec (TOFMS) by providing a voltage pulse to deflect low mass ions and avoid suppression of signals of the larger ions, thus resulting in discrete peaks for each species of ISF product. The appropriate time for the voltage pulse depends on the size of the largest ion species of interest and can conveniently be adjusted to maximize the mass resolution for such ion species.

The ISF products are readily separated according to molecular weight by TOFMS, which orders the species according to their molecular weight. Since the mass of each nucleotide is known, this allows determination of the nucleotide sequence of the nucleic acid analyte by simple comparison of the molecular weights of the consecutive species in the ordered array.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows TOFMS spectrum for nucleotides N to N-18 for a 20-mer oligonucleotide sequenced using a preferred embodiment of the method according to the invention. The "w" ions are marked and the molecular weight assignments are shown over each peak.

Figure 2 shows TOFMS spectrum for nucleotide N-19 for the same oligonucleotide shown in Figure 1.

Figure 3 shows TOFMS spectrum for a 15-mer PS all DNA oligonucleotide. The "w" ions are marked and the molecular weight assignments are shown over each peak.

Figure 4. MALDI-TOF mass spectrum of a 17-mer PS/PO oligodeoxynucleotide (ODN) chimera. In addition to "a-B" (marked by *), "w" and "d" ions, the "a" ions are also present.

Figure 5. MALDI-TOF mass spectrum of a 25-mer PS ODN.

Figure 6. MALDI-TOF mass spectrum of a 20-mer PS ODN with 2'-O-methylation on the sugar units of the first two and last four nucleosides.

Figure 7. MALDI-TOF mass spectrum of a 18-mer PS/MP chimera.

Figure 8. RP-HPLC chromatograms of a neat 25-mer PS ODN (upper trace) and a mixture of the 25-mer PS ODN and a 17-mer PS ODN (lower trace). The mixture was separated into 5 fractions as indicated.

Figure 9. MALDI-TOF mass spectra of the 4 of the 5 fractions separated by RP-HPLC.

Figure 10. Proposed fragmentation pathway for modified oligonucleotides (MONs) containing negatively charged backbone linkage.

Figure 11. Proposed fragmentation pathway for modified oligonucleotides containing neutral backbone linkage.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to the determination of nucleotide sequences for nucleic acids and their analogs. The patents and publications cited herein are known to those skilled in this field and are hereby incorporated by reference in their entirety.

The invention provides an analytical method for determining the nucleotide sequence of nucleic acid analytes, including any chemically modified oligonucleotides. This new method utilizes matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) with delayed pulsed ion extraction. This method is extremely rapid and acts directly on the nucleic acid analyte. It is effective for a variety of nucleic acid analytes, including any chemically modified oligonucleotides which have not previously been successfully sequenced.

The invention provides a method for determining the nucleotide sequence of nucleic acid analytes comprising providing a suitable target for matrix-assisted laser desorption/ionization, such target comprising a nucleic acid analyte in a matrix suitable for such matrix-assisted laser desorption/ionization; irradiating the target with laser irradiation suitable for creating ISF products from the nucleic acid analyte; delaying pulsed ion extraction for a time sufficient to reduce formation of PSD ion fragments; ordering the ISF products according to molecular weight by time-of-flight mass spectrometry; and determining the nucleotide sequence of the nucleic acid analyte by comparing the molecular weights of the ordered ISF products.

Preferably, the suitable target will include a solid support, such as the commercially available solid supports provided by Bruker Analytical Systems (Billerica, MA) or PerSeptive Biosystems (Cambridge, MA). Preferred solid supports include stainless steel and polymer coated metals. Generally, the target is prepared by applying to the solid support a solution comprising the nucleic acid analyte and a matrix suitable for matrix-assisted laser desorption/ionization (MALDI), then allowing the solution on the solid support to evaporate to dryness.

The nucleic acid analyte can be a naturally occurring or synthetic polynucleotide or oligonucleotide, including oligonucleotides having chemically modified internucleoside linkages, sugar backbones or nucleoside bases. For purposes of the invention, the term "oligonucleotide" includes polymers of two or more deoxyribonucleoside, ribonucleoside or 2'-O-substituted ribonucleoside monomers, or any combination thereof. Preferably, such oligonucleotides will have from about 2 to about 100 monomers, and most preferably from about 13 to about 40. Such monomers may be coupled to each other by any of the numerous known internucleoside linkages. In certain preferred embodiments, these internucleoside linkages may be phosphodiester (PO), phosphotriester, phosphorothioate (PS), or phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/ or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines and adamantane. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with an -O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, *e.g.*, with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group.

The suitable matrix can be selected from the matrices known in the art to be suitable for MALDI, and includes a cation exchange resin, preferably in ammonium form. In a preferred embodiment, the cation exchange resin is a 200-400 mesh in ammonium form. Preferred matrices include, without limitation, 3-hydroxypicolinic acid, 3-hydroxypicolinic acid/picolinic acid, 3-hydroxypicolinic acid/sucrose, 2,5-dihydroxybenzoic acid, sinapinic acid, alpha-cyano-4-hydroxy-cinnamic acid, anthranilic acid, 6-aza-2-thiothymine, nicotinic acid, 2,4,6-trihydroxybenzoic acids, 2,4,6-trihydroxyacetophenone and combinations of the same. Other suitable matrices are identified in Fitzgerald *et al.*, Anal. Chem. 65: 3204 (1993). In one

particularly preferred embodiment, the matrix is 3-hydroxypicolinic acid/N-(3-indolylacetyl)-1-leucine (3-HPA/IAL).

The target can be irradiated with any of a variety of laser irradiation sources. Preferred laser irradiation sources include, without limitation, UV lasers, visible lasers and IR lasers. Particularly preferred UV lasers include a 337 nm N_2 laser, a 266 nm laser and a 193 nm laser. One particularly preferred laser irradiation source is the 337 nm N_2 laser provided with the MALDI unit produced by Bruker Analytical Systems. The laser power is sufficient to efficiently produce ISF products without saturating the detector system. To achieve this power level with the Bruker MALDI unit, a laser attenuation level of from about 40 to about 50 is used to give an irradiance of 1-2 107W/cm2. For other types of attenuation systems, attenuation levels of from about 10 to about 110 may be advantageously used. The laser attenuation level can be measured using a power meter (Laser Probe, Inc., Utica, NY, USA). The ion source of the mass spectrometer is opened so that the energy detector could be placed in the laser beam path in the ion source region. The energy output of the laser is recorded at a series of the neutral density attenuator settings.

Several parameters may be automatically set on the MALDI-TOFMS unit, such as on the unit produced by Bruker Daltonics. Preferably, the voltage on the target (acceleration voltage) will be from about +/- 3-30 kV (for positive or negative polarity). In one particularly preferred embodiment, such acceleration voltage will be about +25 kV. In another particularly preferred embodiment, such acceleration voltage will be about +20 kV. The voltage on the auxiliary plate after switching (P2) is preferably from about +/- 2-27 kV. In one particularly preferred embodiment, such P2 voltage is about +22.5 kV. The lens voltage is preferably from about +/- 0-15 kV. In one particularly preferred embodiment, such lens voltage is about +9.2 kV. The voltage on the dual-microchannel-plates detector is preferably from about +/- 1.3-2.0 kV. In one particularly preferred embodiment, such detector voltage is about +1.5 kV. The time elapsed between laser firing and voltage switching on the auxiliary plate is preferably from about 100 to about 400 ns. In one particularly

preferred embodiment, such elapsed time is about 250 ns. The number of spectra summed may preferably range from about 1 to about 1000. In one particularly preferred embodiment, such number will be about 80. The vacuum in the sample chamber region is preferably at or below 5×10^{-6} torr. In one preferred embodiment, such vacuum is 5×10^{-7} torr.

The pulsed ion extraction is delayed for a time sufficient to increase mass resolution of the ISF products, and to reduce PSD ion formation sufficiently to improve the profile of the time-of-flight mass spec (TOFMS) by providing a voltage pulse to deflect low mass ions and avoid suppression of signals of the larger ions, thus resulting in discrete peaks for each of the ISF products. The MALDI-TOFMS unit produced by Bruker is available with the MS modified as to allow delay of the pulsed ion extraction following laser irradiation. The appropriate voltage and time for the ion deflection voltage pulse depends on the size of the largest ion species of interest and can conveniently be adjusted to maximize the mass resolution for such ion species. Preferably, such ion deflection voltage will range from about 0 to about 10 kV. In one particularly preferred embodiment, such ion deflection pulse will be about +2 kV. Preferably, such time will be from about 0-10 microseconds. In one particularly preferred embodiment, such time is about 1-2 microseconds.

The ISF products are readily separated according to molecular weight by TOFMS, which orders the products according to their molecular weight. The observed ISF products include the "w" and "d" ions, and also the "a" and "z" ions. As is understood in the art, the w and z ions are generated by fragmentation of the sequence from the 3' end and the a and d ions are generated by fragmentation of the sequence from the 5' end. Since the mass of each nucleotide is known, this allows determination of the nucleotide sequence of the nucleic acid analyte by simple comparison of the molecular weights of the consecutive species in the ordered array.

The w ions together with the protonated molecular ions often allows the determination of the complete nucleotide sequence. However, when a sequence contains modified nucleotides, at times the w ions alone are insufficient to

determine the entire sequence, and in these cases the d, a and z ions provide supplemental information which allows the construction of the complete nucleotide sequence. Further the presence of the d, a, and z ions also facilitates the determination of the backbone linkage. Thus, for the first time, the sequence and backbone linkage of an unknown sequence which includes modified nucleotides can be determined using a single technique.

Results of such fragment ordering and molecular weight assignment are shown in Figures 1, 2, 3, 4, 5, 6 and 7. The results correctly identified the nucleotide sequence of a 20-mer phosphorothioate oligonucleotide having 2 2'-O-methyl nucleosides at its 5' end and 4 2'-O-methyl nucleosides at its 3' end, Figures 1 and 2, and the nucleotide sequence of a 15-mer phosphorothioate deoxyribonucleotide, Figure 3. The results correctly identified the nucleotide sequence of a 17-mer phosphorothioate/phosphodiester (PS/PO) chimeric oligonucleotide, Figure 4. The results correctly identified the nucleotide sequence of a 25-mer phosphorothioate oligonucleotide, Figure 5. The results correctly identified the nucleotide sequence of a 20-mer phosphorothioate oligonucleotide having 2'-O-methylation on the sugar units of the first two and last four nucleosides, Figure 6. The results correctly identified the nucleotide sequence of a 18-mer phosphorothioate/ methylphosphosphonate (PS/MP) chimeric oligonucleotide, Figure 7. The generality of this method is illustrated by the successful sequencing of four distinct modified oligonucleotides as representative compounds.

The following examples illustrate certain preferred embodiments of novel methods for sequencing of oligonucleotides, in particular modified oligonucleotides. Using the method according to the invention, the ISF occurring in the MALDI process leads to the generation of sequence ions of modified oligonucleotides allowing a rapid determination of their sequences. While the fragmentation pattern appears to be little affected by the modification on the ribose ring of a nucleotide, a greater effect has been observed when modification occurs on the backbone linkages. In general, "w" ions provide the most extensive sequence information. The utility of the accompanying "a", "d" and "z" ions varies according to the type of

backbone modification. For PO ODN and MP ODN, useful confirmation or supplemental information comes from "d" ions. In contrast, "a" ions give the most useful supplemental information for PS ODN. Because the sequencing method of the invention involves no chemical or enzymatic reaction, it is by far the most general sequencing method for small antisense ODNs. Its other advantages include the ability to obtain information on the modification groups and speed; a sequence can be determined in less than 1 minute rather than hours.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to limit the scope of the invention.

Example 1

Preparation of a matrix suitable for MALDI

3-hydroxypicolinic acid (3-HPA; Aldrich Chemical Co., Milwaukee, WI; 10.0 +/- 0.5 mg) was weighed and put into a 1.5 ml Eppendorf tube. To the Eppendorf tube was added 75 microliters HPLC grade water (J.T. Baker) and 75 microliters HPLC grade acetonitrile (EM Science, Gibbstown, NJ). The tube was capped and fixed onto the platform of a Vortex Gene 2 (Scientific Industries, Inc. Bohemia, NY) vortexer, which was operated at vibration level 6 for 5 minutes. N-(3-indolylacetyl)-1-leucine (IAL; Aldrich; 2.0 +/- 0.4 mg) was weighed and put into a 1.5 ml Eppendorf tube, to which was added the freshly prepared 3-HPA solution at such volume as to result in a final IAL concentration of 16 g/l. The Eppendorf tube containing the 3-HPA/IAL was then vortexed for 1 minute. Cation exchange resin in ammonium form (Bio-Rad Laboratories, Richmond, CA; 200-400 mesh; 25 +/- 1 mg) was weighed and added to the Eppendorf tube containing the 3-HPA/IAL solution. The tube was then placed in a heating bath at 50°C for 10 minutes. The tube was then removed from the bath and allowed to cool at room temperature for 2 minutes prior to use. Storage for future use is in darkness.

Example 2

Preparation of an exemplary MALDI target

An 20-mer oligonucleotide phosphorothioate having two 5' terminal 2'-O-methyl nucleosides and four 3' terminal 2'-O-methyl nucleosides was prepared as a solution in HPLC grade water (Baker) at a concentration of 5000 ppm. Four microliters of the matrix prepared according to Example 1 was transferred to an Eppendorf tube, to which two microliters of the oligonucleotide solution was added. To the tube was then added 3 mg of cation exchange resin in ammonium form (200-400 mesh), and the tube was then vortexed for 10 seconds. The Eppendorf tube was allowed to stand briefly, then 1 microliter of the clear solution was withdrawn and deposited on a stainless steel target (Bruker). The solvent was then allowed to evaporate at ambient conditions.

Example 3

Sequence determination of a 20-mer phosphorothioate oligonucleotide nucleic acid analyte using MALDI-TOFMS with delayed pulsed ion extraction

The MALDI target prepared according to Example 2 was placed in the sample chamber of a Bruker Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer equipped to allow delay of pulsed ion extraction following laser irradiation. The instrument settings were as follows: (1) acceleration voltage = +25 kV; (2) P2 voltage = +22.5 kV; (3) lens voltage = +9.2 kV; (4) detector voltage = +1.5 kV; (5) time elapsed between laser firing and voltage switching on the auxiliary plate = 250 ns; (6) laser attenuation level = 40-50; (7) number of spectra summed = 80; (8) ion deflection voltage and deflection pulse duration: a +2 kV pulse was maintained for 2 microseconds to obtain a spectrum for "w" ions comprising N-18 to N for the oligonucleotide (see Figure 1); the same voltage pulse was maintained for 1 microsecond to obtain a spectrum for the "w" ion N-19 (see Figure 2). The MS was externally calibrated with a set of calibration constants generated separately from a known compound under the same conditions. Molecular weight assignments were made for each peak, from which the predicted oligonucleotide sequence was deduced.

Further, the modification on the 2' position of the ribose ring by O-methylation appears to have little effect on backbone fragmentation. When fragmented by MALDI, the 20-mer PS ODN with six 2'-O-methylated nucleotides in the sequence, two on the 5'-end and four on the 3'-end, gave four ion series (Figure 6). As in the case of the 25-mer PS ODN described below in Example 5, "a", and "w" ions are the dominant sequence ions. Additional fragment ion series include "d" and "z" (see Tables 1 and 2). Because the modification occurs to the nucleosides, each residue mass used to assign terminal nucleosides is increased by 30 μ into 238 (C), 239 (T), 263 (A), and 278 (G), respectively. Similarly, the residue mass for each nucleotide containing a 2"-O-methyl group is 335 (C), 350 (T), 359 (A), and 375 (G). The terminal nucleotides containing a 2'-O-methyl group should have the following m/z in the positive ion mode, 354 (C), 369 (T), 378 (A), and 394 (G).

Table I. Measured and theoretical m/z of the "w" ions of the 20-mer all PS ON

ion type	obsd. m/z	theor. m/z	mass diff.	base assign.
$(M+H)^+$	6605	6605		
W_{19}	6364	6365	240	°U
W_{18}	5992	5990	375	°G
W_{17}	5646	5645	345	G
W_{16}	5299	5299	346	G
W_{15}	4955	4954	345	G
W_{14}	4649	4649	305	C
W_{13}	4329	4328	321	T
W_{12}	4009	4008	320	T
W_{11}	3679	3679	329	A
W_{10}	3373	3374	305	C
$(M+2H)^{2+}$	3303	3303		
W_9	3069	3069	305	C
W_8	2749	2748	321	T
W_7	2429	2428	320	T
W_6	2084	2083	345	G
W_5	1779	1778	305	C
W_4	1434	1432	346	G
W_3	1074	1073	359	°A
W_2	714	714	359	°A
W_1	378	378	336	°C
			378	°A

Table 2. Measured and theoretical m/z of the "a", "d", and "z" ions of the 20-mer all PS ON

ion type	obsd. m/z	theor. m/z	mass diff.	base assign.
a_{19}	6231	6228		
a_{18}	5895	5893	336	°C
a_{17}	5533	5534	362	°A
a_{16}	5174	5174	359	°A
a_{15}	4829	4829	345	G
a_{14}	4523	4524	306	C
a_{13}	4178	4178	345	G
a_{12}	3858	3858	320	T
a_{11}	3537	3538	321	T
a_{10}	3232	3233	305	C
a_9	2927	2927	305	C
a_8	2599	2598	328	A
a_7	2278	2278	321	T
a_6	1959	1958	319	T
a_5	1654	1652	305	C
a_4	1308	1307	346	G
a_3	963	962	345	G
a_2	618	617	345	G
d_{19}	6341	6342		
d_{13}	4293	4292		
d_{12}	3970	3972	323	T
d_{11}	3650	3652	320	T
d_9	3040	3041		
d_8	2712	2712	328	A
d_7	2392	2392	320	T
d_6	2072	2072	320	T
d_5	1768	1766	304	C
d_4	1423	1421	345	G
d_3	1074	1076	349	G
d_2	731	731	343	G
z_9	2954	2955		
z_8	2635	2634	319	T
z_7	2314	2314	321	T
z_6	1970	1969	344	G
z_5	1664	1664	306	C
z_4	1319	1318	345	G
z_3	960	959	359	°A
z_2	600	600	360	°A

Example 4

Sequence determination of a 17-mer PS/PO chimeric nucleic acid analyte using MALDI-TOFMS with delayed pulsed ion extraction

A 17-mer PS/PO chimeric oligodeoxynucleotide MALDI target was placed in the sample chamber of a Bruker Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer equipped to allow delay of pulsed ion extraction following laser irradiation. The flight path is 1.6 m. The desorption and ionization was effected with a 337 nm N_2 laser. The irradiance was set at an estimated value of 1-2 x $10^7 W/cm^2$ to achieve a laser attenuance level of 40-50, by measuring the laser energy using a power meter. To improve the signal-to-noise, typically 100 laser shots were summed. Other instrument settings were as follows: (1) acceleration voltage = +20 kV; (2) P2 voltage = +22.5 kV; (3) lens voltage = +9.2 kV; (4) detector voltage = +1.5 kV; and (5) time elapsed between laser firing and voltage switching on the auxiliary plate = 250 ns. Matrix ions were deflected to avoid saturation of the dual-microchannel-plated detector. For fragment ions with a mass to charge ration (m/z) less than 1500, the deflection duration was set at 1000 ns, while for those with m/z above 1500 the deflection duration was set at 2000 ns. To reduce the systematic error introduced by the use of delay-extraction, a mass spectrum was calibrated in two segments. In the lower mass region, a matrix ion peak and the doubly protonated molecular ion were used as the calibrants. In the higher mass region, the singly and doubly protonated molecular ions were used as the calibrants.

Figure 4 shows a mass spectrum of a 17-mer oligodeoxynucleotide consisting of both PS and PO nucleotides. Prominent ion series observable in the mass spectrum include "d" and "w" ion series. Also prominently present in the lower m/z region of the mass spectrum are the "a" ions (Table 3). Because the measurement was taken in positive ion mode, all ions should have two extra protons compared to their negative ion analogs, assuming they are all even electron species. For simplicity the same notation for the same ion type is used regardless the ion polarity. The presence of a complete set of w ions (w_1-w_{16}) provides for

ready sequence determination. Confirmation of the 17-mer sequence can be made by a simple comparison between the expected and experimentally determined m/z of "w" ions (Table 3).

The presence of prominent "a" ions in a MALDI-TOF mass spectrum is a feature that has not been reported for mixed base ODNs before. Equally unusual is the presence of some of the a_n-B(T) (the type and sequence position of the nucleobase cleaved is denoted by the letter in the bracket and an integer number n). Although a_n-B ions are commonly observed in a MALDI-TOF mass spectrum of ODNs, the loss of thymine has been generally considered a unfavored fragmentation pathway (Hillenkamp, Smith). Noticeably missing are the "y" ions, except for y14, y15, and y16. These three "y" ions are likely the remaining failure sequences because the limited efficiency of RP-HPLC in ODN purification.

A sequence can be constructed from the "w" ions together with $(M+H)^+$ ions. The sequence is then compared with segments of sequences constructed from other ion series for verification. In general, this is a more useful strategy because it can be used to determine an unknown sequence. In such an approach, the first step of the sequence determination is to find the w_{k-1} peak (k being the total number of the nucleotides in a given sequence). Because the difference in m/z between $(M+H)^+$ and w_{k-1} corresponds to the mass of a 5'-terminal nucleoside residue, it should match one of the five possible values for unmodified deoxynucleoside, 209 (C), 210 (U), 224 (T), 223 (A), and 249 (G). The m/z difference between two adjacent "w" ions, on the other hand, should correspond to the mass of a nucleotide residue. Thus the w_{k-2} is located by finding the peak having a m/z smaller by one nucleotides the residues mass are 289 (C), 304 (T), 313 (A), 329 (G). These become 305 (C), 320 (T), 329 (A), and 345 (G) for phosphorothioate. The remaining "w" ions except w_1 can be determined successively this way. Being an terminal ion, w_1 has a mass larger by that of H_2O than its corresponding residue mass of a repeating unit. Additional correction due to the ion polarity requires the addition of one proton in the positive ion mode. Therefore, PS w_1 can have the following masses: 324 (C), 339 (T), 348 (A), and 364 (G); PO w_1 has smaller masses by 16 μ.

For the 17-mer in Figure 4, although sequence determination could be achieved with the "w" ion series alone, other type of ions provide useful confirmation. Because the use of high laser irradiance caused peak broadening even with the use of delayed-extraction, the w_{13} ion peak at m/z 4212 is not well separated from the peak at m/z 4205. The w_{12} peak at m/z 3910 is also weak. This affects the assignment of nucleotides at position 4, 5, and 6 (counting from the 5' end). However, information can be unambiguously obtained from d3 (m/z 979), d4 (m/z 1308), d5 (m/z 1613), and d6 (m/z 1942). As shown in Table 3, the partial sequence constructed from these ions match the corresponding sequence constructed from "w" ions. Because w_1 appears in the region where matrix ions are very abundant, there may be cases when the former is overwhelmed by the latter. In such a case, the "d_{k-1}" could give the identity of the 3' terminal nucleoside, as does the "w_{k-1}" the 5' terminal nucleoside. Alternative to the "d" ions, the "a" ions could also give unambiguous sequence assignment for position 4-6 when "w" ions could not. The presence of both "a" and "d" ions could also be very useful in determining the structure of backbone linkage. When a backbone linkage in question is flanked by both "a" and "d" ion peaks, its identity can be revealed from the m/z difference of the two type of ions. In such a case, a m/z difference of 98 indicates a PO linkage, while that of 114 indicates PS linkage.

Table 3. Measured and theoretical m/z of the sequence ions from the PS-PO chimera

ion type	obsd. m/z	theor. m/z	mass diff.	base assign.
$(M+H)^+$	5427	5427		
w_{16}	5193	5194	234	A
w_{15}	4872	4873	320	sT
w_{14}	4544	4544	329	G
w_{13}	4216	4215	328	G
w_{12}	3910	3911	306	T
w_{11}	3582	3581	328	G
w_{10}	3252	3252	330	G
w_9	2922	2923	329	G
w_8	2619	2619	304	T
w_7	2290	2290	329	G
w_6	1961	1960	330	G
w_5	1631	1631	329	G
w_4	1327	1327	304	T
w_3	997	998	330	G
w_2	668	669	329	G
w_1	338	339	330	G
			338	sT
a_9	2807	2809		
a_8	2504	2505	304	T
a_8-G	2354	2354		
a_7	2175	2176	329	G
a_6	1846	1846	329	G
a_6-G	1695	1695		
a_5	1517	1517	329	G
a_5-T	1391	1391		
a_4	1213	1213	304	T
a_4-G	1061	1062		
a_3	884	884	329	G
a_3-G	732	733		
a_2	554	555	330	G
d_{15}	4856	4856		
d_{10}	3236	3235	330	G
d_9	2907	2906	331	G
d_7	2274	2273		
d_6	1944	1943	330	G
d_5	1615	1614	329	G
d_4	1311	1310	304	T
d_3	982	981	329	G
d_2	653	652	330	G

Example 5

Sequence determination of a 25-mer PS oligodeoxynucleotide using MALDI-TOFMS with delayed pulsed ion extraction.

A 25-mer PS oligodeoxynucleotide was sequenced by MALDI-TOFMS using the instrument settings described in Example 4 above. Figure 5 shows a mass spectrum of a 25-mer PS ODN. A complete set of "w" ions can be identified (Table 4). Accompanying the "w" ions are the "a" ions giving a distinct pattern of paired peaks in the mass spectrum (Table 5). Other ion series with generally lower abundances, such as "b", "c", "d", and "z" ions have also been found (Table 6). As shown, the replacement of the non-bridging oxygen atom by a sulfur atom enhances the generation of "a" ions. While only partial "a" ion series is observable in the PS/PO chimera described in Example 4, an almost complete "a" ions sequence (a_2-a_{24}) is observed here. The a_1 ions could no be identified because of the presence of an abundant matrix ion peak at the same m/z position in the spectrum. Besides the prominence of the "a" ions, another feature that appears to be general for PS ODN is that the peaks of "w" ions are larger than those of "a" ions of the same length. Also noteworthy is the presence of "z" ion series hitherto unreported in the MALDI TOFMS literature. Interestingly, "a-B" ions are missing. Following the strategy discussed in the previous section, one compete and three partial sequences can be constructed based on the four types of ion series. The sequences are consistent with one another.

Table 4. Measured and theoretical m/z of the "w" ions of the 25-mer all PS ODN

ion type	obsd. m/z	theor. m/z	mass diff.	base assign.
$(M+H)^+$	7777	7777		
$(M-C)^+$	7685	7666	112	cyt
W_{24}	7567	7568	208	C
W_{23}	7249	7248	319	T
W_{22}	6943	6943	306	C
W_{21}	6623	6623	320	T
W_{20}	6317	6317	306	C
W_{19}	5975	5972	342	G
W_{18}	5667	5667	308	C
W_{17}	5337	5337	330	A
W_{16}	5032	5032	305	C
W_{15}	4726	4727	306	C
W_{14}	4423	4422	303	C
W_{13}	4092	4092	331	A
$(M+2H)^{2+}$	3890	3889		
W_{12}	3772	3772	317	T
W_{11}	3468	3468	304	C
W_{10}	3146	3146	322	T
W_9	2842	2842	304	C
W_8	2521	2521	321	T
W_7	2216	2216	305	C
W_6	1895	1895	321	T
W_5	1589	1590	306	C
W_4	1284	1284	305	C
W_3	963	963	321	T
W_2	643	643	320	T
W_1	339	339	304	C
			339	T

Table 5. Measured and theoretical m/z of the "a" ions from the 25-mer all PS ODN

ion type	obsd. m/z	theor. m/z	mass diff.	base assign.
a_{24}	7440	7439		
a_{23}	7134	7134	306	C
a_{22}	6815	6814	319	T
a_{21}	6495	6493	320	T
a_{20}	6188	6188	307	C
a_{19}	5882	5883	306	C
a_{18}	5562	5563	320	T
a_{17}	5257	5257	305	C
a_{16}	4936	4937	321	T
a_{15}	4631	4632	305	C
a_{14}	4312	4312	319	T
a_{13}	4006	4006	306	C
a_{12}	3687	3686	319	T
a_{11}	3357	3357	330	A
a_{10}	3052	3052	305	C
a_9	2746	2746	306	C
a_8	2441	2441	305	C
a_7	2111	2112	330	A
a_6	1805	1807	306	C
a_5	1460	1461	345	G
a_4	1154	1156	306	C
a_3	833	832	321	T
a_2	528	527	305	C

Table 6. Measured and theoretical m/z of the "b", "c", "d", and "z" ions of the 25-mer all PS ODN

ion type	obsd. m/z	theor. m/z	mass diff.	base assign.
b_{24}	7456	7457		
b_{23}	7154	7152	305	C
c_5	1556	1556		
c_4	1250	1250	306	C
c_3	930	929	321	T
d_{24}	7553	7553		
d_{23}	7249	7248	304	C
d_{22}	6928	6928	321	T
d_{21}	6606	6608	322	T
d_{20}	6302	6302	304	C
d_{19}	5997	5997	305	C
d_{17}	5372	5372		
d_{16}	5053	5051	319	T
d_{15}	4745	4746	308	C
d_{13}	4121	4120		
d_{12}	3799	3800	322	T
d_{10}	3166	3166		
d_9	2861	2860	305	C
d_8	2556	2555	305	C
d_7	2225	2226	331	A
d_6	1920	1921	305	C
d_5	1574	1575	346	G
d_4	1269	1270	305	C
d_3	948	950	321	T
d_2	642	645	306	C
z_{10}	3034	3032		
z_9	2728	2728	306	C
z_8	2406	2407	322	T
z_7	2102	2102	304	C
z_6	1780	1781	322	T
z_5	1475	1476	305	C
z_4	1169	1170	306	C
z_3	849	849	320	T

Example 6

Sequence determination of a 18-mer PS/MP chimeric oligodeoxynucleotide using MALDI-TOFMS with delayed pulsed ion extraction.

A 18-mer PS/MP chimeric oligodeoxynucleotide was sequenced by MALDI-TOFMS using the instrument settings described in Example 4 above. Besides phosphorothioate, another popular modified backbone structure is methylphosphonate. The substitution of the non-bridging oxygen atom by a methyl, as opposed to a sulfur atom for ODNs, reduces the fragmentation efficiency (Figure 7). A gap appears in the methylphosphonate region. This proves to be extremely valuable as they fill the sequence gap. The residue masses for methylphosphonate deoxynucleotide are 287 (C), 302 (T), 311 (A), and 327 (G).

Example 7

To investigate the origin of the ISF Products the 25-mer PS ODN was mixed with a 17-mer PS-ODN of the same sequence nested on the 3'-end in a molar ratio of 100:1. When analyzed by RP-HPLC, the two PS ODN appear as two separate components (Figure 8). Fractionation of the mixture at time points indicated in the chromatogram gave 5 fractions. Analysis by MALDI-TOFMS revealed that Fraction A consisted mostly of the 17-mer. Due to low the concentration, the signal of the 17-mer is low (data not shown). The MALDI-TOFMS mass spectra of the other fractions are shown in Figure 9. In Fraction B besides the 17-mer no other accompanying fragments are present. As expected, the latter eluting fractions contain decreasing amount of the 17-mer but increasing amount of the 25-mer. Importantly, none of these fractions showed the presence of the fragment ions. In contrast, Fraction E, having the highest concentration of the 25-mer, gave all the fragment ions (Fig. 10), Note the 17-mer is not present in the spectrum. If the fragments were present prior to MALDI, Fraction B is expected to contain not only the 17-mer, but also some of the fragments. Conversely, if the 17-mer could be removed from Fraction E, the fragments should also have been removed from the 25-mer if they had been present. We can therefore conclude that the fragment ions did not originate from species that are present prior to MALDI; rather, it is the result of MALDI.

Example 8

The influence of experimental conditions

No qualitative difference has been observed when the 25-mer PS ODN, shown in Figure 5, was analyzed in either the positive or negative polarity, using 3-HPA/IAL as the matrix. As in the positive polarity, the dominant ion series are "w" and "a" ions. Additional ion series include "d" and "z" ion, the latter appear only in the lower m/z region. The tendency to arc in the ion source at high laser irradiance makes the positive polarity a better choice for routine analysis.

Although the signal to noise ratio (S/N) of the fast fragment ions can be improved as the irradiance increases from the threshold irradiance, which is defined as the irradiance necessary to give a S/N of 2 for a given fragment ion, the abundance of the fragment ions relative to that of the $(M+H)^+$ ion of the 17-mer PS ODN did not scale linearly with the irradiance applied at the sample. At 6×10^6 W/cm^2, only "w_3" - "w_{12}" were observed. Increasing the irradiance by 30% made all "w" ions and most of the "a" ions observable. However, from this point on further increase in irradiance actually decreased the relative abundance of smaller "w" ions (smaller than w_{10}), while little affecting that of the larger "w" ions.

The delay time affects the absolute intensity of all ions. However, the abundance of fragment ions relative to that of $(M+H)^+$ remained little changed within 200 - 400 ns.

Example 9

Proposed fragmentation pathway for modified oligonucleotides

Because "a-B" ions of ODNs are usually present in a MALDI-TOF mass spectrum, most of the ion fragmentation mechanisms which have been proposed involve the cleavage of the base, followed by the cleavage of the 3' glycosidic bond. However, using the method according to the invention, a PS ODN could give prominent depurinated products while showing little sign of "a-B" ions. The

observation suggests that there is another fragmentation pathway that is independent of the base cleavage. The m/z values of the "a" and "w" ions suggest that the cleavage involves at least one hydrogen transfer. The transfer could occur directly from the "a" precursor to the "w" precursor or involve the matrix species, as the cleavage happens in an area where matrix ions and radicals are present. However, the observation that PS ODNs have greater tendency than PO or MP ODNs to form "w" and "a" ions is not in favor of a cleavage mechanism involving matrix species. Instead, the observation is better explained by a mechanism involving E1 type *syn* elimination (Lowry, T, H., Richardson, K. S. Mechanism and theory in organic chemistry, Harper & Row: New York, 1981, pp 530-556). In this mechanism, the 4' hydrogen is transferred to the negatively charged non-bridging oxygen or sulfur atom resulting in the formation of a double bond between 3' and 4' positions and the cleavage of the glycosidic bond (Figure 10). The replacement of the non-bridging oxygen with a neutral methyl group would impede the process. In such a case, the 5' PM group could orient itself to participate in the elimination of the 4' hydrogen leading to the formation of a double bond between the 4' and 5' positions or a rearranged sugar ring (Figure 11).

What is claimed is:

1. A method for determining the nucleotide sequence of a nucleic acid analyte, the method comprising:

a) providing a suitable target for matrix-assisted laser desorption/ionization, such target comprising a nucleic acid analyte in a matrix suitable for such matrix-assisted laser desorption/ionization;

b) irradiating the target with laser irradiation suitable for creating in-source fragmentation products from the nucleic acid analyte;

c) delaying pulsed ion extraction for a time sufficient to increase mass resolution of the in-source fragmentation products and to reduce formation of PSD ion fragments;

d) ordering the in-source fragmentation products according to molecular weight by time-of-flight mass spectrometry; and

e) determining the nucleotide sequence of the nucleic acid analyte by comparing the molecular weights of the in-source fragmentation products.

2. The method of claim 1 wherein said nucleic acid analyte further comprises a naturally occuring or synthetic oligonucleotide or polynucleotide.

3. The method of claim 2 wherein said oligonucleotide further comprises an oligonucleotide having at least one chemically modified internucleoside linkage, chemically modified sugar backbone or chemically modified nucleoside base.

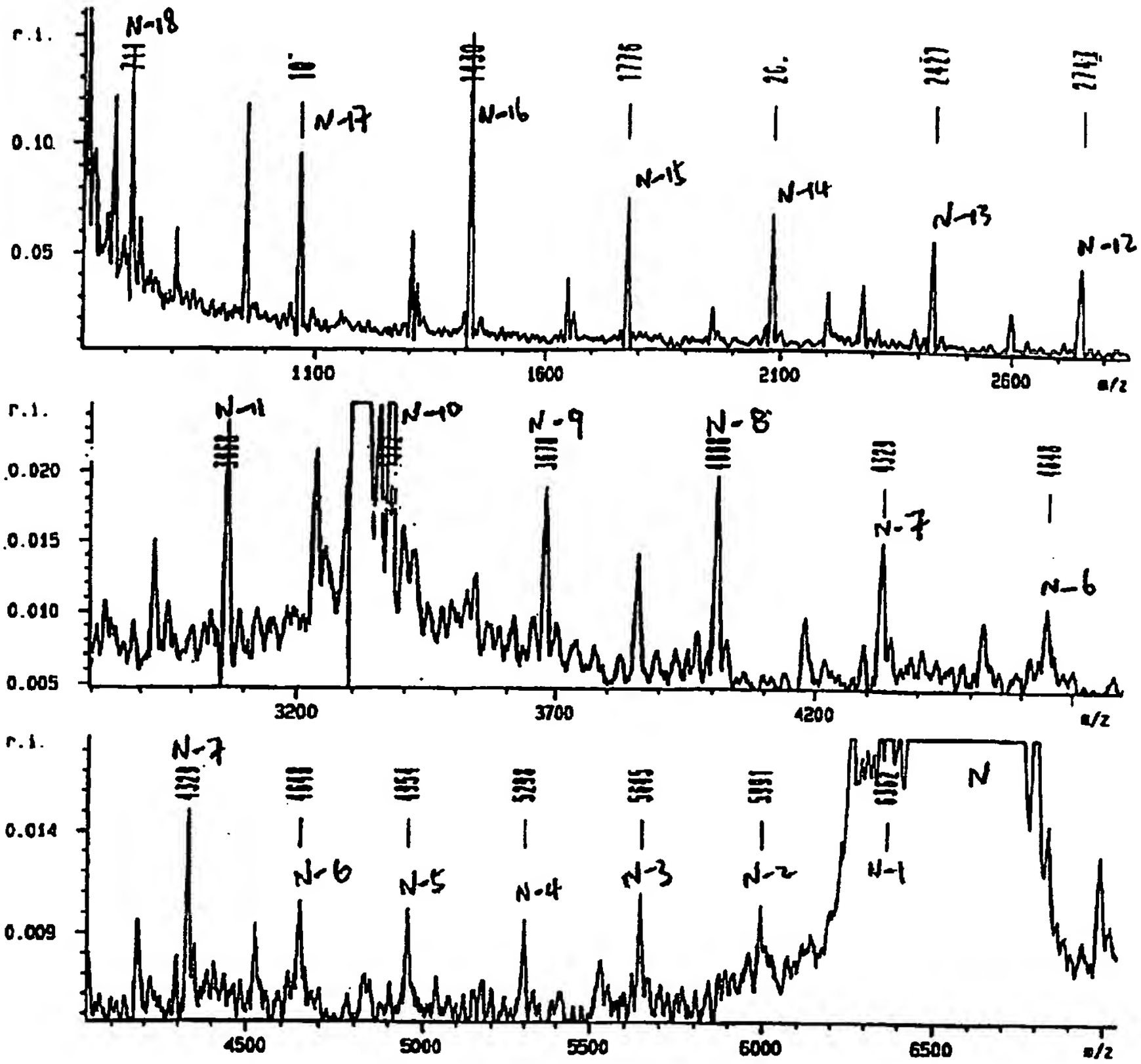
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Fig. 1

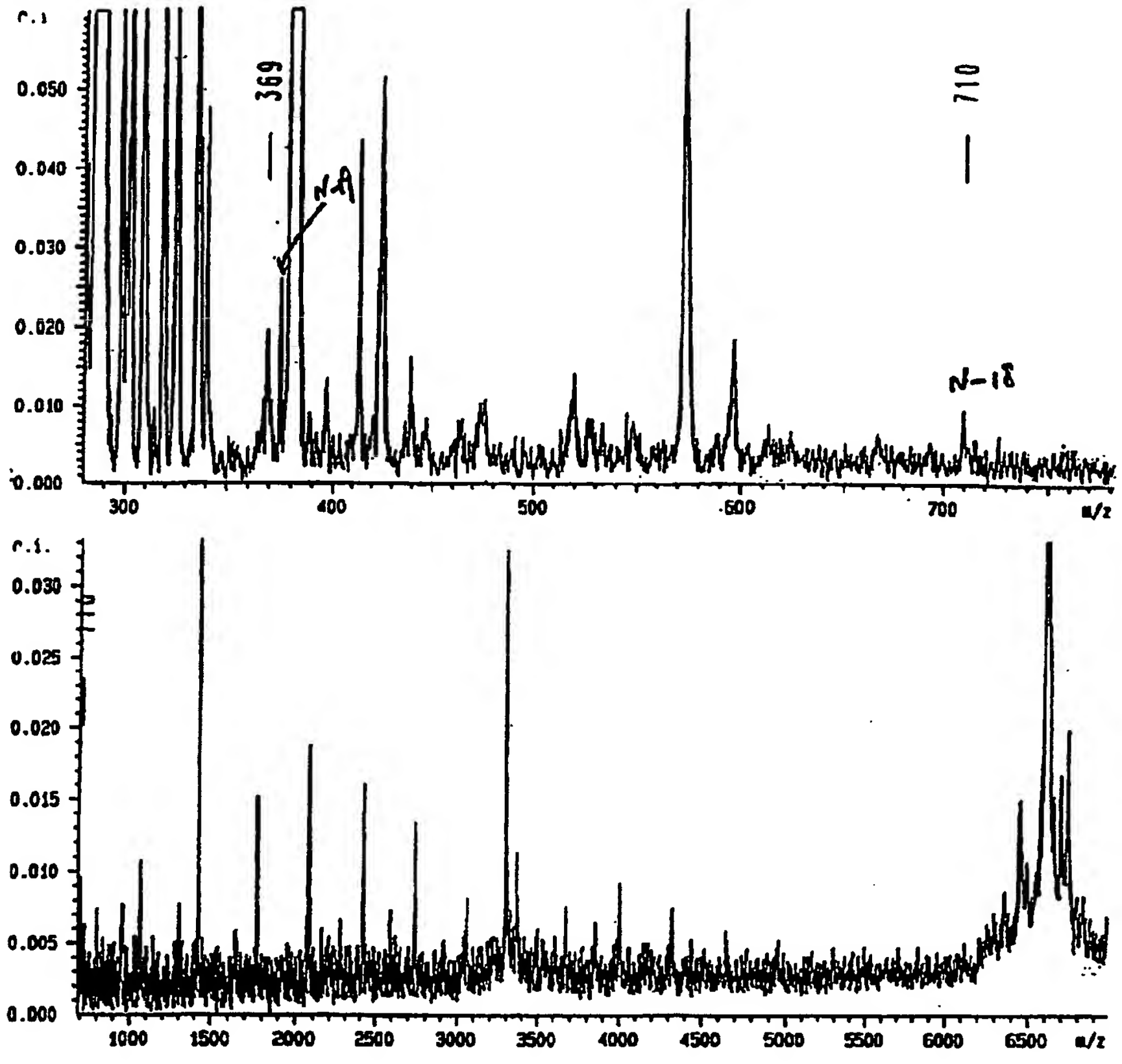
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Fig. 2

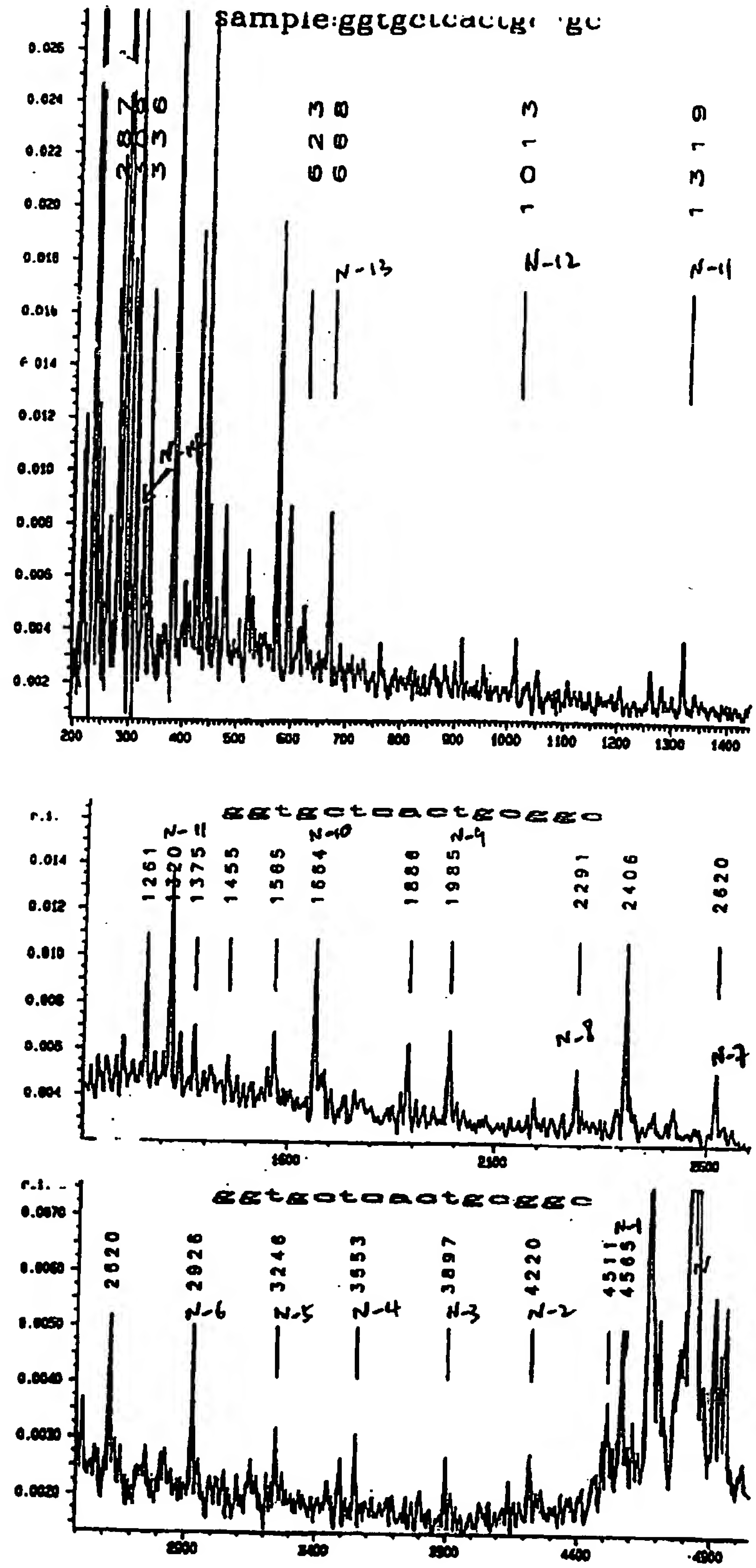
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Fig. 3

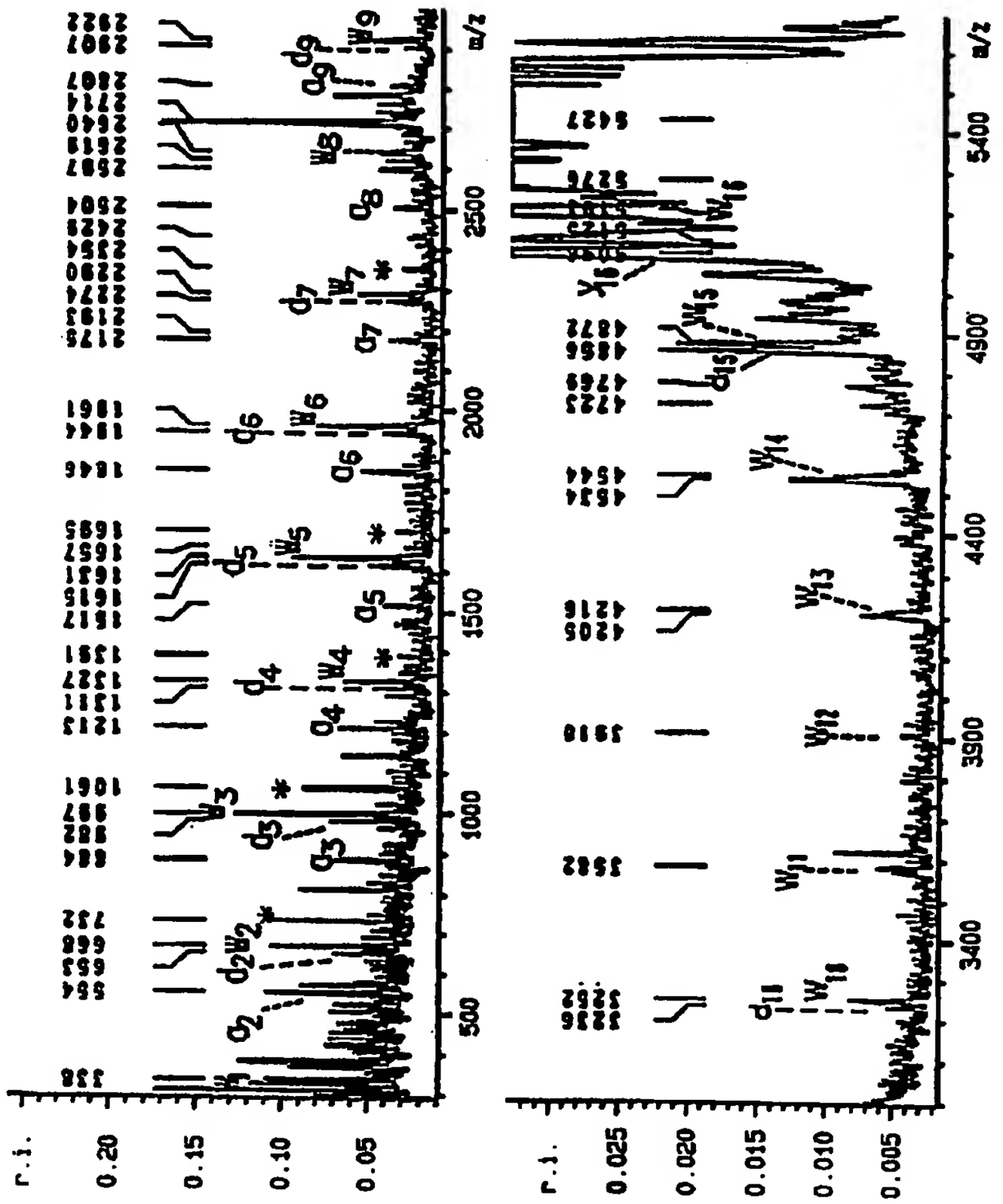
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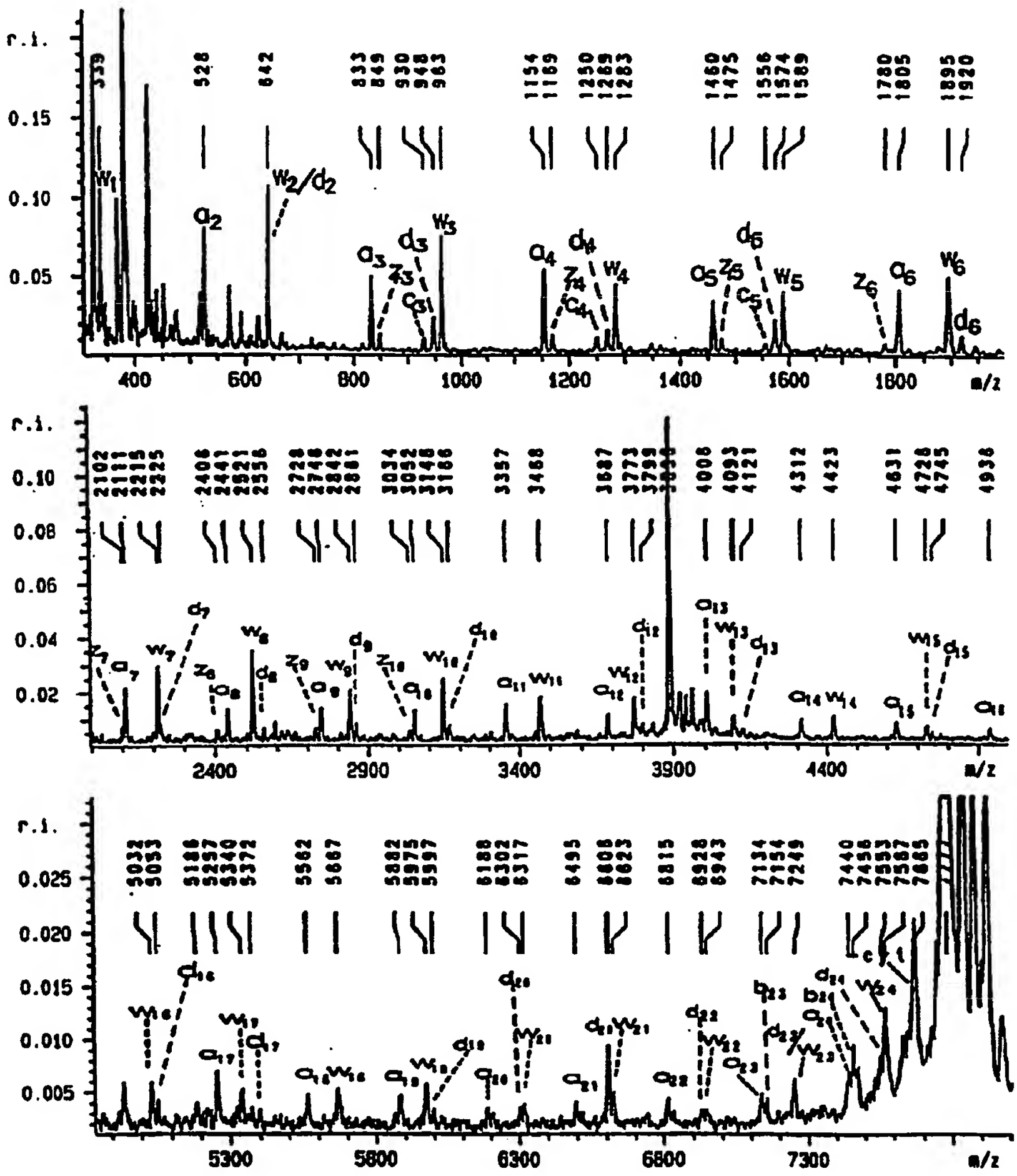
Figure 4

Figure 5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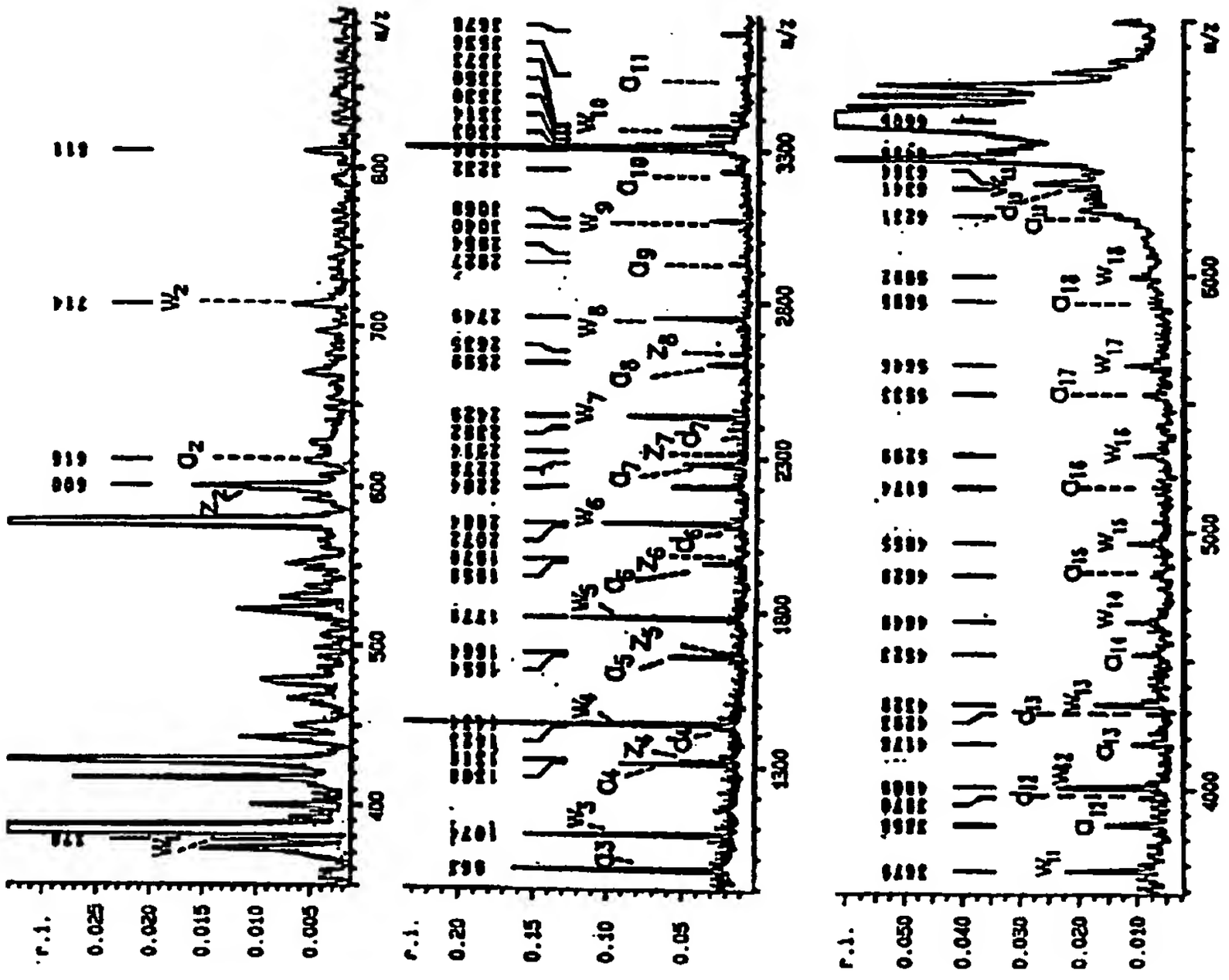
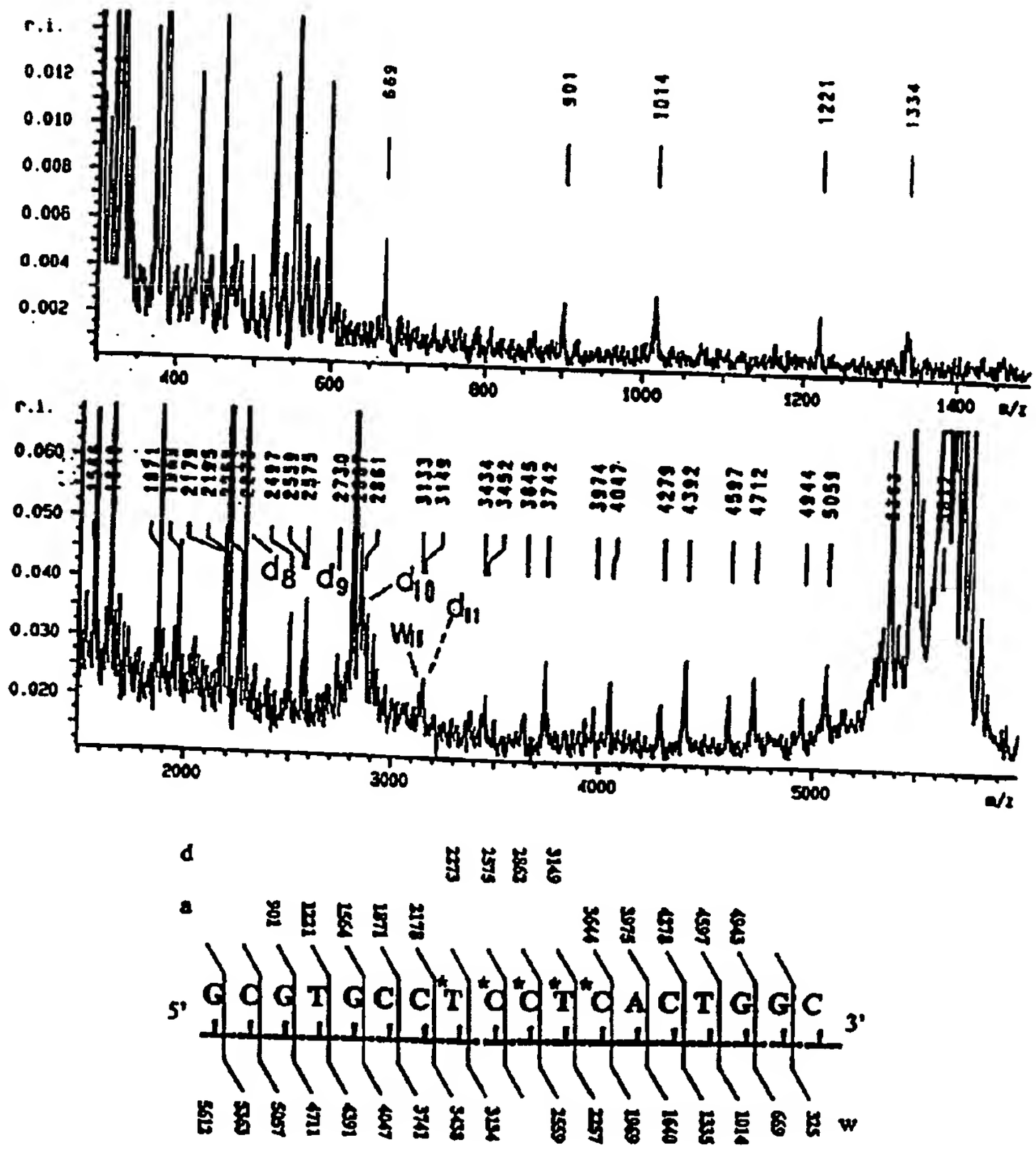


Figure 6

Figure 7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1000
1200
1400
m/z
r.i.
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0.050
0.040
0.030
0.020
d8
d9
d10
d11
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m/z

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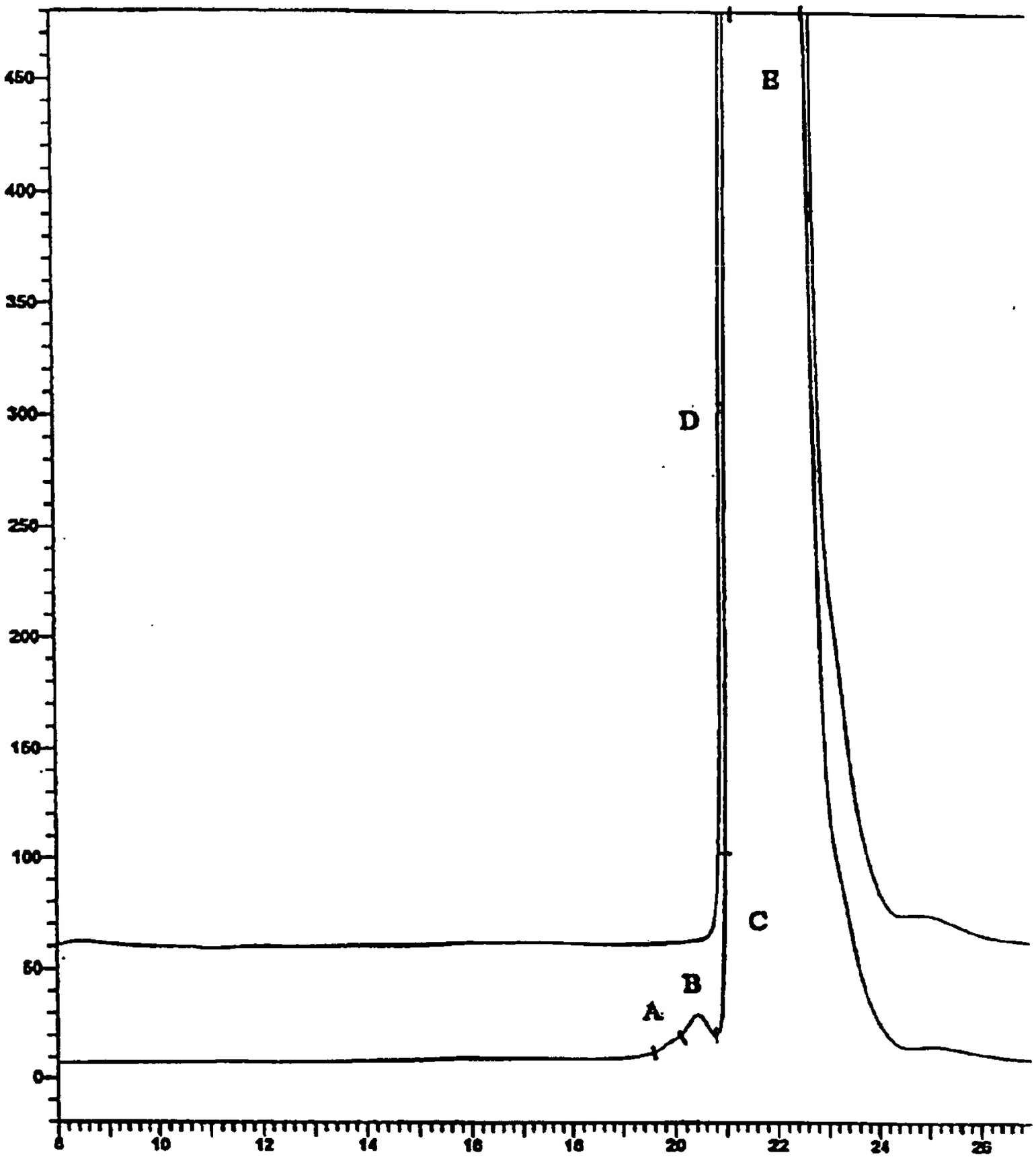
3'
w

Figure 8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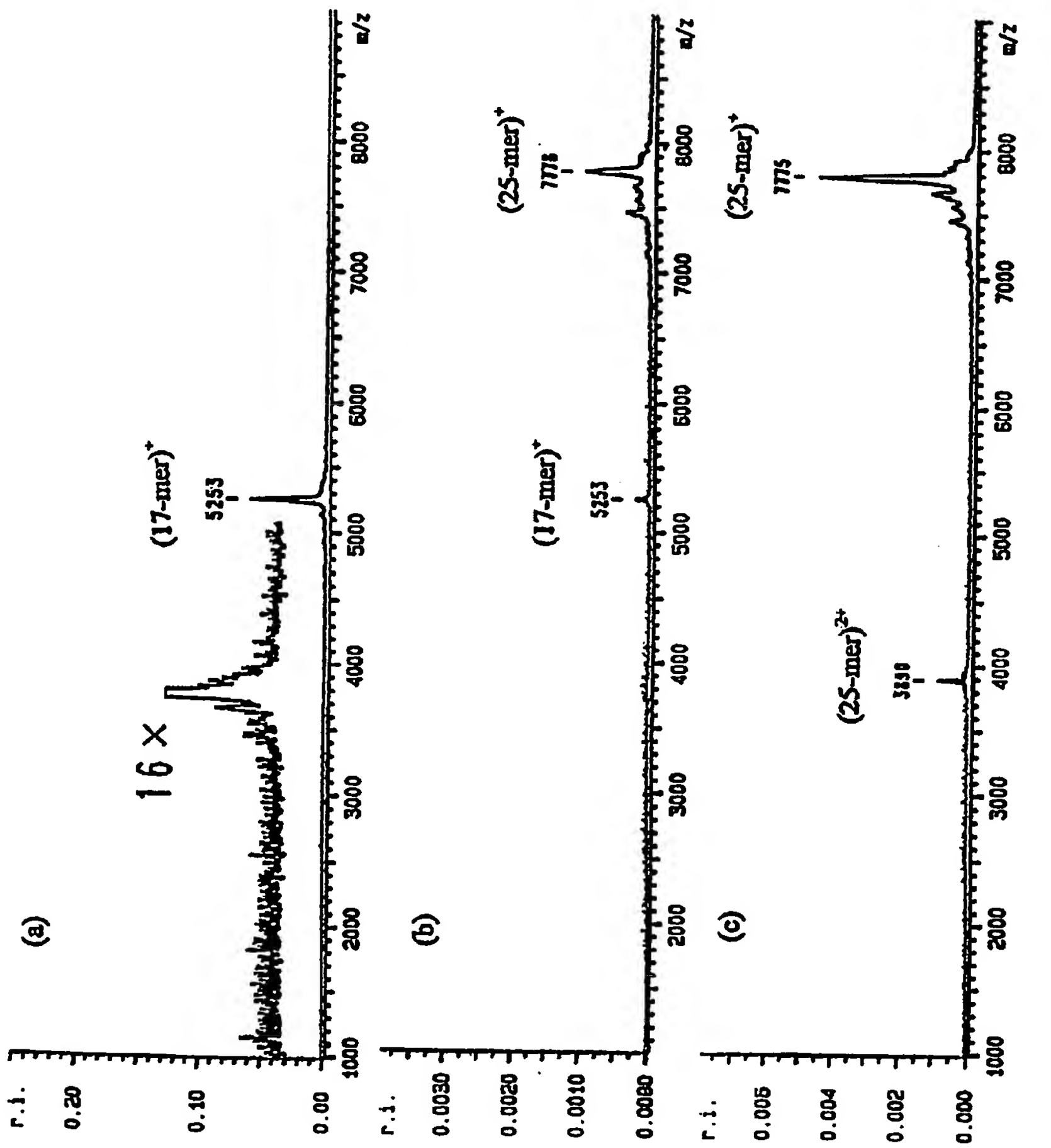
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3000
4000
5000
6000
7000
8000
m/z

Figure 9

Figure 10

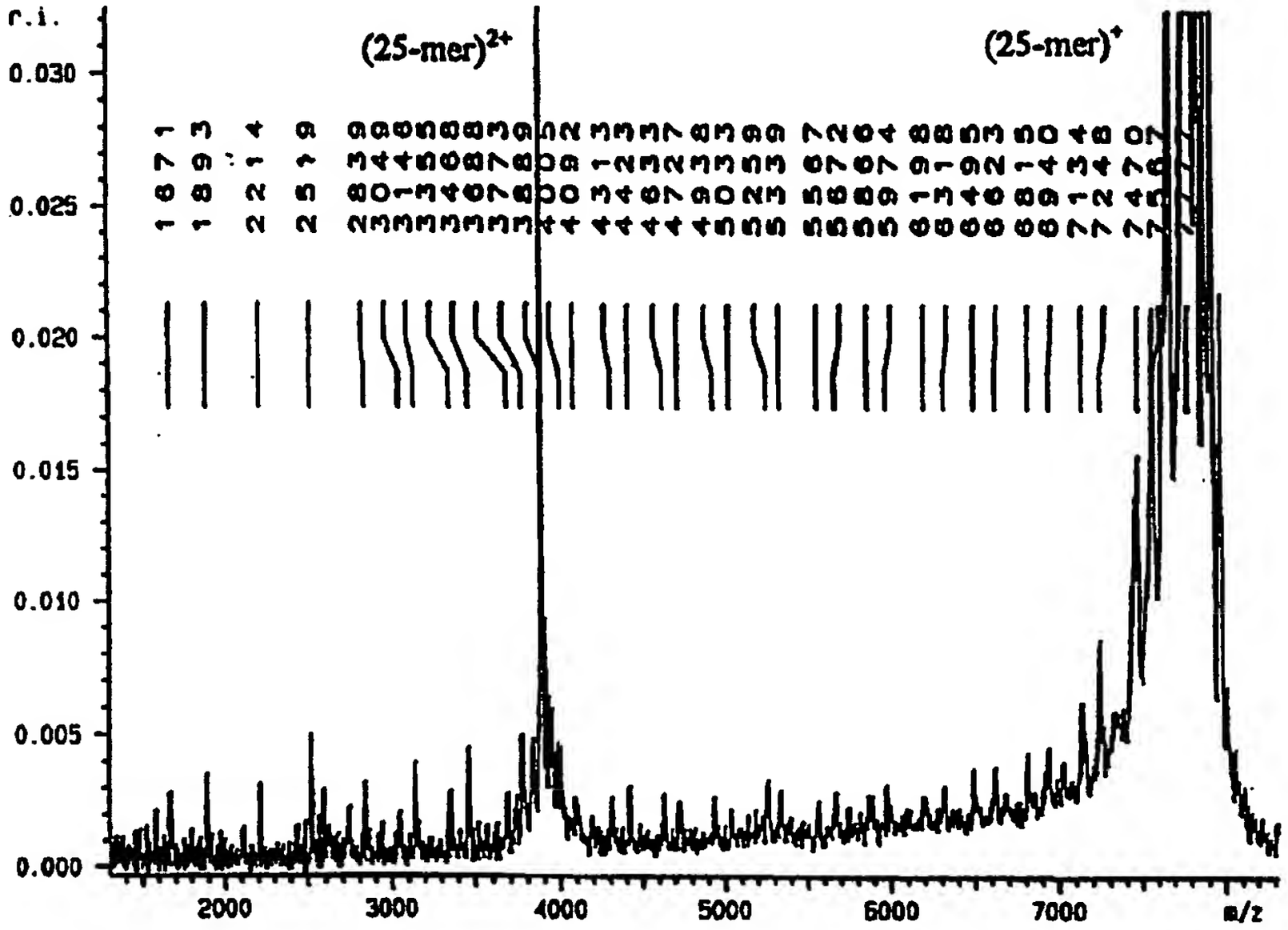

r.i.
0.030
0.025
0.020
0.015
0.010
0.005
0.000
(25-mer)2+
(25-mer)+
2000
3000
4000
5000
6000
7000
m/z

Figure 11

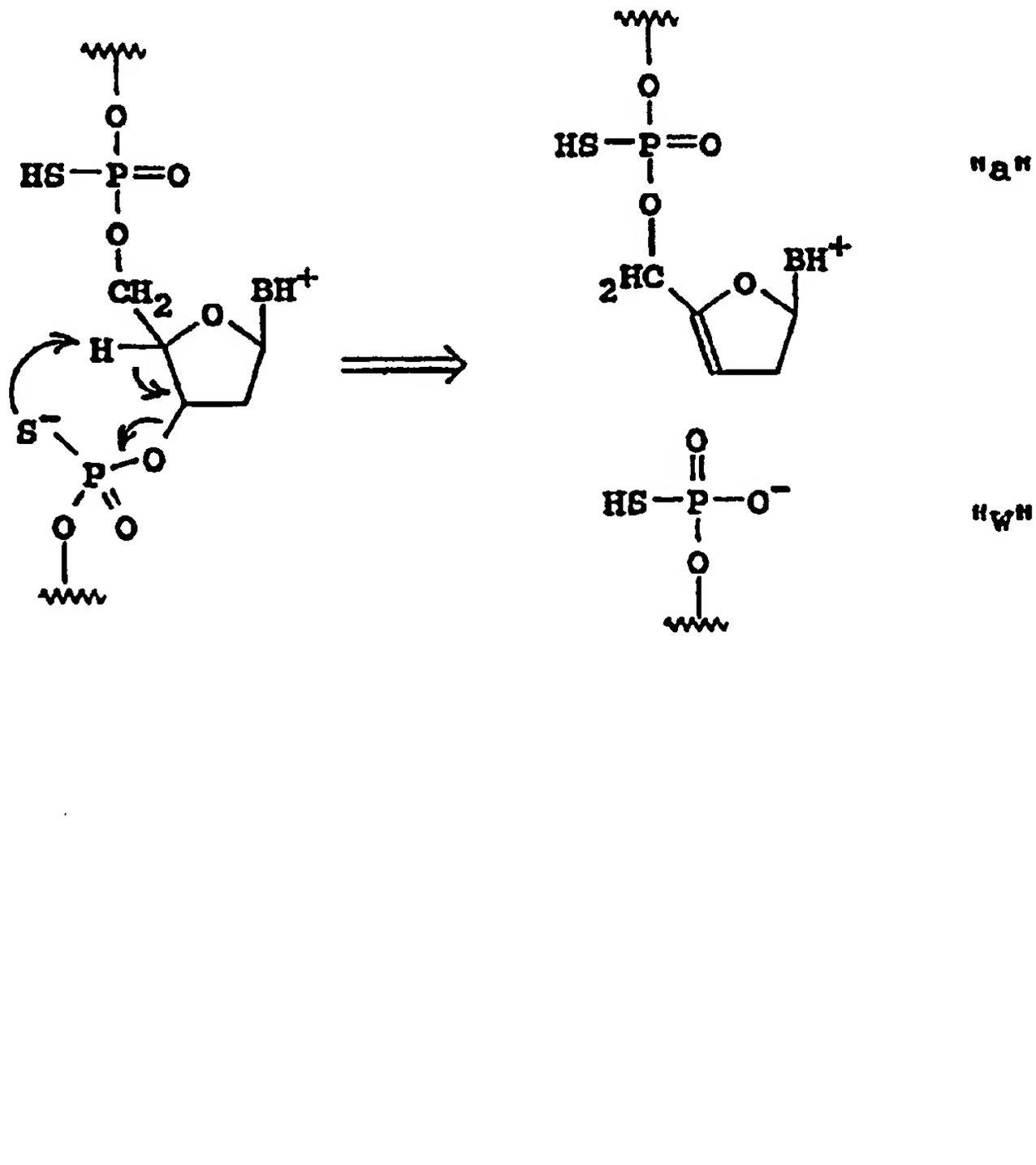
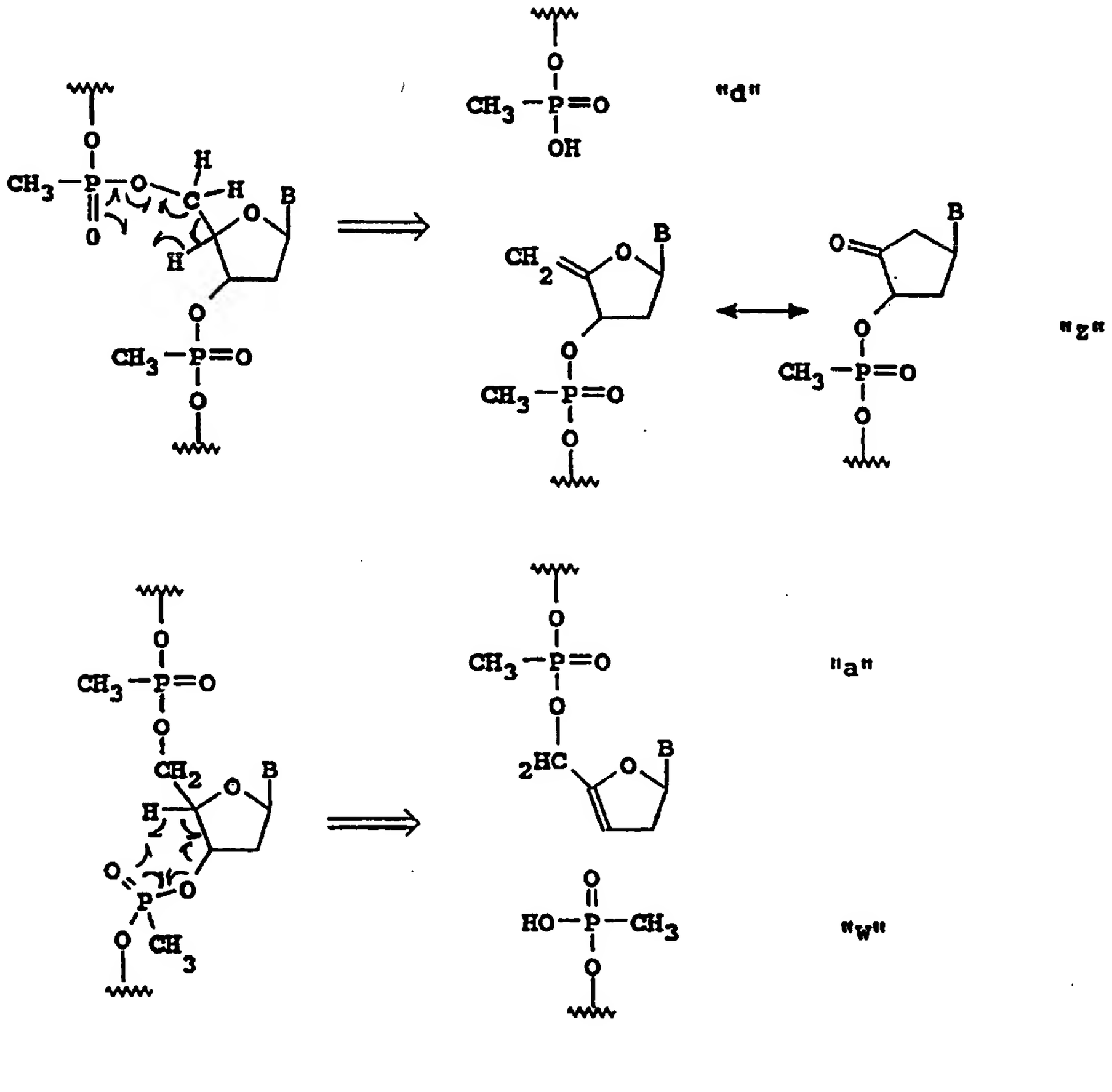


Figure 12



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/12688

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 H01J49/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JUHASZ ET AL.: "Applications of DE MALDI TOF MS to oligonucleotide analysis" ANALYTICAL CHEMISTRY., vol. 68, no. 6, March 1996, COLUMBUS US, pages 941-946, XP002047877 see page 945 - page 946 ---	1-3
Y	NORDHOFF ET AL.: "Direct mass spectrometric sequencing of low picomole amounts of oligodeoxynucleotides with up to 21 bases by MALDI MS" J. MASS SPECTROMETRY, vol. 30, January 1995, pages 99-112, XP002047878 cited in the application see the whole document ---	1-3

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 1997	11/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/12688

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BROWN ET AL.: "Sequence specific fragmentation of matrix assisted laser desorbed protein/peptide ions" ANALYTICAL CHEMISTRY., vol. 67, November 1995, COLUMBUS US, pages 3990-3999, XP002047879 cited in the application see the whole document	1-3
Y	ROSKEY ET AL.: "DNA sequencing by delayed extraction MALDI TOF mass spectrometry" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, May 1996, WASHINGTON US, pages 4724-4729, XP002047880 see the whole document	1-3
Y	SMIRNOV I P ET AL: "SEQUENCING OLIGONUCLEOTIDES BY EXONUCLEASE DIGESTION AND DELAYED EXTRACTION MATRIX-ASSISTED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY" ANALYTICAL BIOCHEMISTRY, vol. 238, no. 1, 15 June 1996, pages 19-25, XP000597084 see the whole document	1-3
A	TALBO ET AL.: "Aspects of the sequencing of carbohydrates and oligonucleotides by MALDI post source decay" RAPID COMMUNICATIONS IN MASS SPECTROMETRY, vol. 10, no. 1, 1996, pages 100-103, XP002047881	
A	WO 92 21140 A (UNIV JOHNS HOPKINS) 26 November 1992	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/12688

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		CA 2103038 A	17-11-92
		DE 69220943 D	21-08-97
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